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RT-PCR-Based Detection of Six Garlic Viruses and Their Phylogenetic Relationships

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Abstract Six viruses of the genera *Carlavirus* (*Garlic mosaic virus*, GarMV, and *Garlic latent virus*, GarLV), *Allexivirus* (*Garlic virus X*, GarV-X, and *Garlic mite-borne filamentous virus*, GarMbFV) and *Potyvirus* (*Leek yellow stripe virus*, LYSV, and *Onion yellow dwarf virus*, OYDV) from Korean garlic plants with mosaic symptoms were simultaneously detected by multiplex RT-PCR and subsequently sequenced. An immunocapture RT-PCR for the detection of GarLV, LYSV, and OYDV was also performed. The coat protein phylogenetic analysis of the garlic viruses showed that the Korean isolates were most closely related to the isolates from China, Japan, Brazil, and Argentina. This study is the first report for the differentiation of six garlic viruses in Korea by simultaneous detection using multiplex RT-PCR.

Key words: Garlic virus, immunocapture RT-PCR, multiplex RT-PCR, phylogenetic analysis, simultaneous detection

Most of the garlic plants cultivated throughout the world are infected with viruses that give rise to mosaic symptoms on leaves [20], including the genera *Carlavirus* (*Garlic mosaic virus*, GarMV, and *Garlic latent virus*, GarLV), *Allexivirus* (*Garlic virus X*, GarV-X, and *Garlic mite-borne filamentous virus*, GarMbFV) and *Potyvirus* (*Leek yellow stripe virus*, LYSV, and *Onion yellow dwarf virus*, OYDV). Double infection by these viruses results in more severe symptoms than those arising from a single infection [2], indicating a possible synergetic relationship between these viruses in the development of virus disease.

Because of its high sensitivity and rapidity, reverse transcription-polymerase chain reaction (RT-PCR) is a molecular method frequently used for the detection of plant viruses and found to be more sensitive than enzyme-linked immunosorbent assay (ELISA) [9, 12, 13]. Immunocapture (IC) RT-PCR has also been developed for the detection of several economically important viruses [7] to simplify sample preparation and reduce the risk of sample contamination [10]. To date, many multiplex RT-PCR assays have been described for the simultaneous detection of two to six viruses in plants, including potato, apple, olive, grapevine, citrus, strawberry, and sugar beet [1, 6, 14–16, 23, 26].

Many RT-PCR assays for garlic viruses have used common primers designed from consensus sequences of the viral genus of interest in China and Japan [3–5, 24, 25]. However, multiplex RT-PCR has not previously been performed to detect six garlic viruses, which are members of three taxonomic groups, with six pairs of primers at a time. In the present study, the amplification of six viruses (GarMV, GarLV, GarV-X, GarMbFV, LYSV, and OYDV) from infected Korean garlic plants was performed by uniplex RT-PCR, multiplex RT-PCR, and IC RT-PCR, and their phylogenetic relationships, based on the amino acid sequences of the coat proteins, are described.

Garlic (*Allium sativum* L.) plant leaves with mosaic symptoms in Korea were obtained from Korean Rural Development Administration. Using six pairs of primers based on the alignment of the virus coat protein genes in the GenBank database, National Center for Biotechnology Information (NCBI) (Table 1), target sequences were amplified by uniplex RT-PCR, multiplex RT-PCR, and IC RT-PCR. The RT reaction using Sensiscript RT kit (Qiagen, Germany) was performed with total RNA from 50–100 mg of infected garlic leaves, as described by Park *et al.* [18]. For IC RT-PCR, leaves (500 mg) of infected garlic plants were ground in extraction buffer [500 mM Tris-HCl (pH 8.2), 140 mM NaCl, 0.7 mM polyvinyl pyrrolidone K25, 20 mM polyethylene

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Table 1. Primers used for the detection of six garlic viruses by RT-PCR.

Virus[a]	Sequence (5'-3')	GenBank accession number[c]	Product size (bp)
GarMV	ATGATGAATTAGATGCAGGC (+)[b] TCACGTCATGTGCTGTGTGC (-)	AF314146 (position 5-829 nt), E03282-3	825
GarLV	TATACAGCGCTCTAAATTGA(+) TTCTCTGTTTGATCAACATC (-)	Z68502 (position 7061-7981 nt)	921
GarV-X	GATCGGAACCAAGGAATAA (+) GAGTGGAAACCATATTCGAG (-)	NC_001800 (position 6881-7541 nt)	661
OYDV	AAGGATAAAGACGTTGATG (+) CGTGTGTTGGTTCTTGTTTA (-)	AF228414 (position 61-753 nt), AF438412, AY170321, AF071226, X95874, AB000837-846, X89402, AB000472, D73378, NC_005029, AJ292223-4, AJ307033, AJ293278, Y11826	693
LYSV	GAACTAGATGCAGGGACAC (+) GCCGTCTAATCCAAACAGC (-)	AF228415 (position 10-777 nt), AF538950, AY007693, AF071525, NC_004011, AJ307032, X89711, D11118, D28590, AB0005610-12	768
GarMbFV	ATGTCAGGTTCCACAAGT (+) TCAGAACGTAATCATGGGA (-)	X98991 (position 37-759 nt), I38892	723

[a]GarMV, *Garlic mosaic virus*; GarLV, *Garlic latent virus*; GarV-X, *Garlic virus X*; OYDV, *Onion yellow dwarf virus*; LYSV, *Leek yellow stripe virus*; GarMbFV, *Garlic mite-borne filamentous virus*.

[b](+), sense primer; (-), antisense primer.

[c]Reference sequences for designing primers that are published on the GenBank database, NCBI.

glycol 6000 and 0.05% Tween 20] and the suspension was centrifuged at 13,000 rpm and 4°C for 10 min. In the tubes coated with anti-SLV (*Shallot latent virus*, synonym: GarLV), LYSV, and OYDV IgG (Agdia, U.S.A.) diluted 1:200 in coating buffer (0.1 N $NaHCO_3$, pH 9.5), the RT reaction was carried out, as described by Sefc *et al.* [19]. The following PCR reaction mixtures contained 40 pmol each of primer (depending on the subsequent PCR), 2.5 mM each of dNTP, 3 unit of *Taq* polymerase (Promega, U.S.A.), and 4–5 μl of cDNA in a final volume of 50 μl. The reaction profile for 35 cycles was as follows: predenaturation at 95°C for 5 min, denaturation at 94–95°C for 1 min 30 s, annealing at 45–50°C for 1 min 30 s, extension at 72°C for 2 min, and final extension at 72°C for 10 min in a Mastercycler Gradient (Eppendorf, Germany). The PCR products were resolved on 2% agarose gels, purified, sequenced by automatic sequencing using the BigDye™ Terminator Cycle sequencing Ready Reaction Kit V.2 (Perkin Elmer, U.S.A.), and analyzed with DNA sequence analysis Version 2.1.1 (Perkin Elmer), as shown by Lee *et al.* [11]. The sequences of the virus coat protein gene from Korean garlic plants were aligned with those of other countries using the CLUSTAL W program [22], and the phylogenetic analysis was performed with the amino acid sequences of the coat proteins using the PAUP program [21].

We designed a multiplex assay in which a total of six primer pairs were used for detecting six viral agents by a one-step PCR reaction. Of the six primers, four primers were based on the coat protein regions of virus isolates, which is a highly conserved region among published virus isolates, except for GarLV and GarV-X whose sequences have been reported in Korea (Z68502 and NC_001800, respectively). The annealing temperature was selected in order to decrease nonspecific priming or other artifacts. Times for annealing and extension were minimized to reduce the possibility of nonspecific amplification. All RT-PCR assays yielded almost equally reliable and sensitive results, producing the expected size of PCR fragments; 825, 921, 661, 723, 768, and 693 bp generated by GarMV, GarLV, GarV-X, GarMbFV, LYSV, and OYDV, respectively (Figs. 1 and 2). This indicated the lack of interference

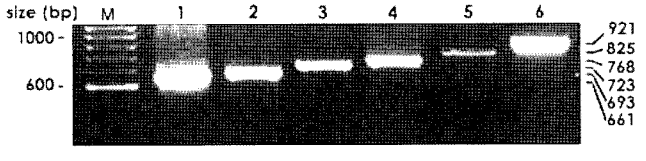

Fig. 1. Uniplex RT-PCR products for garlic viruses.
Lanes M, 100 bp ladder (Invitrogen, U.K.); 1, GarV-X; 2, OYDV; 3, GarMbFV; 4, LYSV; 5, GarMV; 6, GarLV.

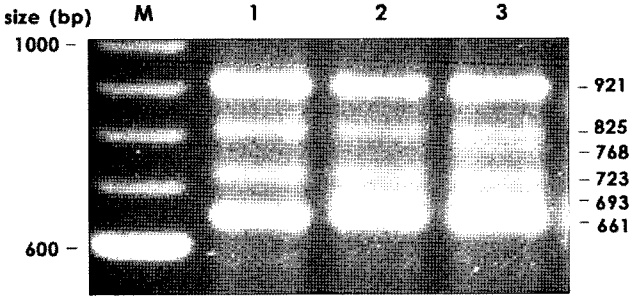

Fig. 2. Multiplex RT-PCR products for garlic viruses.
Lanes M, 100 bp ladder; 1, GarMV, GarV-X, GarMbFV, and GarLV; 2, GarMV, GarV-X, OYDV, GarMbFV, and GarLV; 3, GarMV, GarV-X, OYDV, LYSV, GarMbFV, and GarLV.

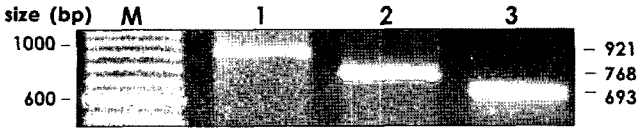

Fig. 3. IC RT-PCR products for garlic viruses.
Lanes M, 100 bp ladder; 1, GarLV; 2, LYSV; 3, OYDV.

among PCR primers during the multiplex reaction, as described by Bertolini *et al.* [1]. As seen in Fig. 2, multiplex RT-PCR was shown to be as reliable and highly sensitive to detect and identify garlic viruses as is uniplex RT-PCR (Fig. 1). Whereas uniplex RT-PCR is potentially expensive and resource intensive, multiplex RT-PCR

Table 2. Amino acid sequence identity (%) between coat proteins of LYSV and OYDV isolates.

(A) LYSV

Isolate[a]	1	2	3	4	5	6	7	8	9	10	11	12	13
1		96	78	82	75	79	79	80	80	78	80	79	79
2			80	84	77	82	82	81	81	80	83	81	82
3				80	89	87	82	81	81	94	80	88	88
4					77	82	82	84	84	78	88	80	80
5						85	85	77	78	93	76	84	84
6							100	80	80	88	81	95	95
7								80	80	88	81	95	95
8									98	79	83	80	80
9										80	83	80	80
10											79	87	87
11												81	81
12													99
13													

(B) OYDV

Isolate[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1		93	89	90	75	90	75	90	93	90	92	93	98	90	75	75	97	97	90	90	90	89	93	91	90
2			90	94	78	93	78	92	95	91	93	99	95	93	77	78	95	95	93	91	93	92	93	92	93
3				89	82	89	82	88	88	93	90	90	89	87	82	82	88	90	89	88	89	90	93	93	89
4					77	91	77	91	94	89	93	93	92	95	77	77	92	92	96	95	96	90	92	90	99
5						77	100	77	81	77	77	77	77	77	95	96	76	77	77	76	77	76	77	78	77
6							77	98	94	90	90	93	91	90	77	78	91	92	90	90	91	90	91	91	90
7								77	81	77	77	77	77	77	95	96	76	77	77	77	77	76	77	78	77
8									93	90	90	93	91	90	77	78	91	92	90	89	91	90	91	91	90
9										91	99	95	95	93	81	81	94	92	95	95	94	93	96	93	93
10											90	90	90	88	70	78	90	92	87	87	88	90	94	98	88
11												92	93	93	77	77	92	93	93	93	92	90	94	91	92
12													94	92	77	77	94	94	92	90	92	92	92	91	93
13														91	76	77	99	99	92	91	91	91	94	93	91
14															77	77	91	91	96	93	95	89	91	89	94
15																99	76	76	70	75	76	76	77	78	77
16																	76	77	77	76	77	76	77	78	77
17																		98	92	90	91	90	93	92	91
18																			92	91	91	92	94	93	92
19																				97	97	90	91	89	95
20																					96	89	90	88	94
21																						90	91	90	95
22																							93	91	89
23																								96	91
24																									89
25																									

[a] **1**, Korea (AY390255); **2**, Brazil (AF228415); **3**, Indonesia (AB005612); **4**, Taiwan (AF538950); **5**, Argentina (AY007693); **6**, Israel (AF071525); **7**, China (NC_004011); **8**, China (AJ307032); **9**, China (AB005610); **10**, Germany (X89711); **11**, UAE (AB005611); **12**, Japan (D11118); **13**, Japan (D28590).

[b] **1**, Korea (AY390253); **2**, Brazil (AF228414); **3**, Brazil (AF438412); **4-8**, Indonesia (AB000841-5); **9**, Italy (AY170321); **10**, Argentina (X95874); **11**, Argentina (AB000837); **12**, Argentina (X89402); **13**, Japan (AB000840); **14**, Japan (AB000838); **15**, Japan (AB000472); **16**, Japan (D73378); **17**, China (AJ292224); **18**, China (AJ293278); **19**, China (NC_005029); **20**, China (AJ292223); **21**, China (AJ307033); **22**, Israel (AF071226); **23**, The Netherlands (AB000836); **24**, The Netherlands (Y11826); **25**, UAE (AB000839).

incorporates different sets of specific primers for two to six targets in one reaction tube and enables simultaneous amplification of different target nucleic acids in a single test, reducing material costs, labor, and time. Furthermore, no cross-reaction was found between the individual primers and non-target virus, as seen in the uniplex RT-PCR. Therefore, the multiplex RT-PCR described here could be a reliable and suitable tool for indexing garlic viruses in garlic crops.

For application for routine diagnostic work, a large-scale evaluation is necessary to compare its efficiency with other molecular biology based techniques. To compare sensitivity and specificity of multiplex RT-PCR with multiplex IC RT-PCR, we performed uniplex and multiplex IC RT-PCR for GarLV, LYSV, and OYDV. As shown in Fig. 3, uniplex IC RT-PCR products were successfully obtained. However, contrary to Jacobi *et al.* [7], multiplex IC RT-PCR failed to simultaneously detect two or three viruses. It might have been due to the cross-reaction in the combination of virus-specific antibody capture and low primer specificity. In addition, antibodies for many garlic viruses are not commercially available. Therefore, the advantages of multiplex RT-PCR for simultaneous detection of six viruses are obvious, as described by Nie and Singh [17].

The partial viral coat protein genes from Korean garlic plants have been sequenced and deposited in the GenBank database: GarMbFV (AY390254), GarMV (AY390256), LYSV (AY390255), and OYDV (AY390253). The sequences of GarLV and GarV-X obtained in this study were well matched with previously reported ones (Z68502 and NC_001800, respectively) in Korea [2, 20, 24]. As described by Chen *et al.* [2], the Korean isolates of GarLV and GarV-X were shown to have higher identity to the isolates from China, demonstrated by the coat protein phylogenetic analysis. The new GarMbFV isolate shared 58% and 75% amino acid sequence identity (63% and 86% in the nucleotide sequence) with Japan (L38892, Japanese garlic virus) and Argentina (X98991) isolates, respectively, indicating that the new Korean GarMbFV isolate is closer to the Argentinian isolate. The newly obtained GarMV coat protein sequence from Korean garlic plants (AY390256) had 85–92% amino acids sequence identity (85–87% nucleotides) to that of China (AF314146) and Japan (E03282-3), but 55–64% amino acids identity (54–62% nucleotides) to that of *Scallion mosaic virus* (NC_003399, China) and *Turnip mosaic virus* (AF071526, Israel), showing that the new Korean GarMV sequence is most closely related to the isolates from China and Japan. The identity between coat protein amino acid sequences of LYSV and OYDV isolates is shown in Table 2(A) and (B), respectively, as shown by Kim and Crow [8]. A phylogenetic tree based on the coat protein amino acid sequences of all LYSV and OYDV isolates is also shown in Fig. 4. The new Korean LYSV and OYDV are discriminated as two distant potyviruses

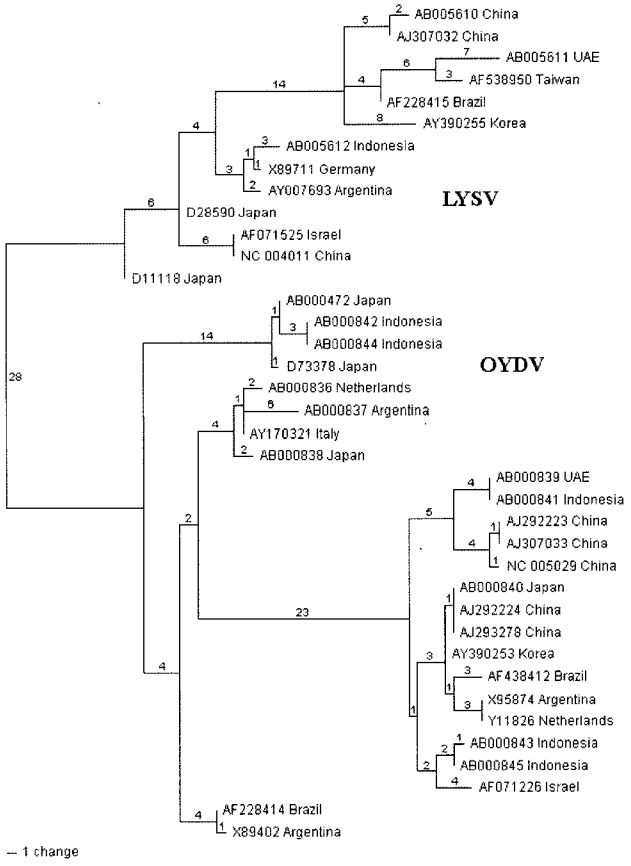

Fig. 4. Phylogenetic analysis of the coat protein amino acid sequences of LYSV and OYDV.
The neighbor-joining method available in PAUP 4.0 was used to construct the phylogenetic tree. The values at the forks indicate the number of times out of 1,000 trees that this grouping occurred after bootstrapping the data. The scale bar shows the number of substitutions per base.

and clustered with those of Brazil, China, and Japan in a neighboring subgroup, which is in agreement with the previously reported data [2, 4]. The result in Fig. 4 confirmed the usefulness of coat protein sequence analysis in assessing the taxonomic relationships among garlic viruses, as described by Tsuneyoshi *et al.* [25].

This is the first report on potyviruses (LYSV and OYDV) and allexivirus (GarMbFV) that infect garlic plants in Korea, indicating that there are multiple groups of garlic viruses co-circulating in Korea. The improved knowledge of multiplex RT-PCR and the molecular characterization of these six garlic viruses will greatly assist further investigation of garlic plant-infecting viruses, control of garlic virus disease, and production of virus-free garlic plants.

Acknowledgments

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